GI 5160C

NOVEL BMP PRODUCTS

INSA1

INSA2

This application is a continuation-in-part of U.S. Serial Nos. 493,272 filed March 14, 1990 (which is a CIP of 406,217 filed September 12, 1989); 378,537 filed July 11, 1989; and 655,579 filed February 14, 1991 which is a divisional of U.S. Serial No. 179,100 filed April 8, 1988 (now U.S. Patent 5,013,649) which is a continuation-in-part of U.S. Serial Nos. 028,285 filed March 20, 1987 now abandoned; 943,332 filed December 17, 1986 now abandoned; and 880,776 filed July 1, 1986 now abandoned.

The present invention relates to a novel family of purified proteins designated BMP-2 and BMP-4 proteins and processes for obtaining and producing them. These proteins may be used to induce bone and/or cartilage formation and in wound healing and tissue repair.

BMP-2 and BMP-4 proteins have previously been referred to collectively as BMP-2 proteins (BMP-2 previously referred to as BMP-2A or BMP-2 Class I and BMP-4 as BMP-2B or BMP-2 Class II).

Human BMP-2 proteins are characterized by an amino acid sequence comprising amino acid #299 (His, Pro, Leu...) - #396 (Arg) of Figure 2 (SEQ ID NO: 4). Human BMP-2 proteins are further characterized as dimers of BMP-2 subunits. Mature BMP-2 is characterized by comprising amino acid #283 (Gln, Ala, Lys...) - #396 (Arg) of Figure 2. Mature BMP-2 is further characterized as a disulfide linked dimer wherein each subunit comprises amino acids #283-#396 of Figure 2 (SEQ ID NO: 4).

Human BMP-2 may be produced by culturing a cell transformed with a DNA sequence comprising the nucleotide coding sequence from nucleotide #356 to #1543 as shown in Figure 2 (SEQ ID NO: 3) and recovering and purifying from the culture medium a protein comprising amino acid #299 to #396 as shown in Figure 2 (SEQ ID NO: 4), substantially free from other

proteinaceous materials with which it is co-produced. Human BMP-2 is characterized by the ability to induce bone formation. Human BMP-2 is further characterized by the ability to induce cartilage formation. Human BMP-2 may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below. In preferred embodiments, the proteins of the invention demonstrate activity in this assay at a concentration of 10 μg - 500 μg/gram of bone. BMP-2 proteins may be characterized by the ability of 1 μg of the protein to score at least +2 in the rat bone formation assay of Example III using the modified scoring method described in Example VII.

The bovine BMP-2 protein is a member of the family of BMP-2 proteins of the invention. Bovine BMP-2 proteins comprise the amino acid sequence represented by amino acid #32 to amino acid #129 of Figure 1 (SEQ ID NO: 2). These proteins are capable of inducing the formation of cartilage and/or bone. Bovine BMP-2 may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below. In preferred embodiments, the proteins of the invention demonstrate activity in this assay at a concentration of 10 μg - 500 μg/gram of bone. These proteins may be characterized by the ability of 1 μg of the protein to score at least +2 in the rat bone formation assay described in Example III using the modified scoring method as described in Example VII.

Human BMP-4 proteins are characterized by an amino acid sequence comprising amino acids #311(His, Ser, Leu ...) - #408 (Arg) as shown in Figure 3 (SEQ ID NO: 6). Mature BMP-4 comprises amino acids #293 (Ser, Pro, Lys...) - #408 (Arg) of Figure 3. BMP-4 proteins are further characterized as dimers of BMP-4 subunits. Mature BMP-4 is further characterized as a disulfide linked dimer wherein each subunit comprises amino acids #293-#408 of Figure 3 (SEQ ID NO: 6).

BMP-4 may be produced by culturing a cell transformed with

a DNA sequence comprising the nucleotide coding sequence from nucleotide #403 to nucleotide #1626 substantially as shown in Figure 3 (SEQ ID NO: 5) and recovering and purifying from the culture medium a protein containing the amino acid sequence from amino acid #311 to #408 as shown in Figure 3 (SEQ ID NO: 6) substantially free from other proteinaceous materials with which it is co-produced. BMP-4 proteins are capable of inducing the formation of bone. BMP-4 proteins are capable of inducing formation of cartilage. BMP-4 proteins are further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below. In preferred embodiments, the proteins of the invention demonstrate activity in this assay at a concentration of 10 μg - 500 μg/gram of bone. These proteins may be characterized by the ability of 1 μg of the protein to score at least +2 in the rat bone formation assay of Example III using the modified scoring method described in Example VII.

Another aspect of the invention provides pharmaceutical compositions containing a therapeutically effective amount of a BMP-2 or BMP-4 protein in a pharmaceutically acceptable vehicle or carrier. These compositions of the invention may be utilized in the formation of cartilage. These compositions may further be utilized in the formation of bone. They may also be used for wound healing and tissue repair. In further embodiments the compositions of the invention may be utilized for neuronal survival.

Further compositions of the invention may comprise a therapeutically effective amount of BMP-2 and BMP-4 in a pharmaceutically acceptable vehicle. Compositions of the invention may further include, in addition to a BMP-2 or BMP-4 protein, at least one other therapeutically useful agent such as the BMP proteins BMP-1, BMP-3, BMP-5, BMP-6, BMP-7, and BMP-8 disclosed respectively in co-owned U.S. patent applications Ser. No. 655,578, Ser. No. 179,197, Ser. No. 370,547, Ser. No. 370,544 Ser. No. 370,549, and Ser. No. 525,357. The

compositions of the invention may comprise, in addition to a BMP-2 or BMP-4 protein, other therapeutically useful agents including growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and transforming growth factor (TGF-α and TGF-β). The compositions may also include an appropriate matrix for instance, for supporting the composition and providing a surface for bone and/or cartilage growth. The matrix may provide slow release of the BMP protein and/or the appropriate environment for presentation of the BMP protein.

The BMP-2 and BMP-4 compositions may be employed in methods for treating a number of bone and/or cartilage defects, periodontal disease and various types of wounds. These methods, according to the invention, entail administering to a patient needing such bone and/or cartilage formation wound healing or tissue repair, an effective amount of a BMP-2 or BMP-4 protein. These methods may also entail the administration of a protein of the invention in conjunction with at least one of the novel BMP proteins disclosed in the co-owned applications described above. In addition, these methods may also include the administration of a BMP-2 or BMP-4 protein with other growth factors.

Still a further aspect of the invention are DNA sequences encoding a BMP-2 or BMP-4 protein. Such sequences include the sequence of nucleotides in a 5' to 3' direction illustrated in Figures 1 through 3 (SEQ ID NO: 1,3, and 5) or DNA sequences which hybridize under stringent conditions with the DNA sequences of Figures 1 - 3 and encode a protein having the ability to induce the formation of cartilage and/or bone. Finally, allelic or other variations of the sequences of Figures 1 through 3, whether such nucleotide changes result in changes in the peptide sequence or not, are also included in the present invention.

A further aspect of the invention entails a vector comprising a DNA sequence as described above in operative association with an expression control sequence therefor. Such

vector may be employed in a novel process for producing a BMP-2 or BMP-4 protein of the invention in which a cell line transformed with a DNA sequence encoding a BMP-2 or BMP-4 protein in operative association with an expression control sequence therefor, is cultured in a suitable culture medium and a BMP-2 or BMP-4 protein is recovered and purified therefrom. This claimed process may employ a number of known cells both prokaryotic and eukaryotic as host cells for expression of the polypeptide.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description and preferred embodiments thereof.

Brief Description of the Drawing

FIG. 1 comprises partial DNA and derived amino acid sequence of bovine BMP-2 from bacteriophage lambda bP-21, ATCC #40310 further described below.

FIG. 2 sets forth the DNA and derived amino acid sequence of human BMP-2 from lambda U2OS-39, ATCC #40345 further described below.

FIG. 3 sets forth the DNA and derived amino acid sequence of human BMP-4 from lambda U2OS-3, ATCC #40342 further described below.

Detailed Description of the Invention

BMP-2 proteins are characterized by an amino acid sequence comprising amino acid #299-#396 of Figure 2 (SEQ ID NO: 4). BMP-2 proteins are further characterized as dimers of BMP-2 subunits. Mature BMP-2 comprises amino acids #283-#396 of Figure 2. Mature BMP-2 is further characterized as a disulfide linked homodimer wherein each subunit comprises amino acids #283-#396 of Figure 2 (SEQ ID NO: 4).

The purified human BMP-2 proteins of the present invention

are produced by culturing a host cell transformed wth a DNA sequence comprising the DNA coding sequence of Figure 2 (SEQ ID NO: 3) from nucleotide #356 to nucleotide #1543 and recovering and purifying from the culture medium a protein which contains the 97 amino acid sequence or a substantially homologous sequence as represented by amino acid #299 to #396 of Figure 2 (SEQ ID NO: 4). The BMP-2 proteins recovered from the culture medium are purified by isolating them from other proteinaceous materials with which they are co-produced and from other contaminants present.

BMP-4 proteins are characterized by an amino acid sequence comprising amino acids #311-#408 as shown in Figure 3 (SEQ ID NO: 6). BMP-4 proteins are further characterized as dimers of BMP-4 subunits. Mature BMP-4 comprises amino acids #293-#408 of Figure 3. Mature BMP-4 is further characterized as a disulfide linked homodimer each subunit comprising amino acids #293-#408 of Figure 3 (SEQ ID NO: 6).

The purified BMP-4 proteins are produced by culturing a host cell transformed with a DNA sequence comprising the DNA coding sequence of Figure 3 (SEQ ID NO: 5) from nucleotide #403 to nucleotide #1626 and recovering and purifying from the culture medium a protein comprising the amino acid sequence from amino acid #311 to #408 of Figure 3 (SEQ ID NO: 6). The BMP-4 proteins recovered from the culture medium are purified by isolating them from other proteinaceous materials with which they are co-produced and from other contaminants present.

BMP-2 and BMP-4 proteins are characterized by the ability to induce the formation of bone. They are further characterized by the ability to induce the formation of cartilage. BMP-2 and BMP-4 proteins may be further characterized by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below. In preferred embodiments, the proteins of the invention demonstrate activity in this rat bone formation assay at a concentration of 10 μg - 500 μg/gram of bone. These

proteins may be characterized by the ability of 1 μg of the protein to score at least +2 in the rat bone formation assay using the modified scoring method described in Example VII.

The BMP-2 and BMP-4 proteins provided herein also include factors encoded by the sequences similar to those of Figures 1 -3 (SEQ ID NO: 1,3,5), but into which modifications are naturally provided (e.g. allelic variations in the nucleotide sequence which may result in amino acid changes in the polypeptide) or deliberately engineered. For example, synthetic polypeptides may wholly or partially duplicate continuous sequences of the amino acid residues of Figures 1 - 3. These sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with bone growth factor polypeptides of Figures 1 - 3 may possess bone growth factor biological properties in common therewith. Thus, they may be employed as biologically active substitutes for naturally-occurring BMP-2 and BMP-4 polypeptides in therapeutic processes.

Other specific mutations of the sequences of BMP-2 and BMP-4 proteins described herein involve modifications of at least one of the glycosylation sites. The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at asparagine-linked glycosylation recognition sites present in the sequences of BMP-2 and BMP-4 proteins shown in Figures 1 - 3. The asparagine-linked glycosylation recognition sites comprise tripeptide sequences which are specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence.

The present invention also encompasses the novel DNA

sequences, free of association with DNA sequences encoding other proteinaceous materials, and coding on expression for BMP-2 and BMP-4 proteins. These DNA sequences include those depicted in Figures 1 - 3 in a 5' to 3' direction and those sequences which hybridize under stringent hybridization conditions [see, T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] to the DNA sequences of Figures 1 - 3 and encode a protein having cartilage and/or bone inducing activity.

Similarly, DNA sequences which code for BMP-2 and BMP-4 polypeptides coded for by the sequences of Figures 1 - 3, but which differ in codon sequence due to the degeneracies of the genetic code or allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) also encode the novel factors described herein. Variations in the DNA sequences of Figures 1 - 3 which are caused by point mutations or by induced modifications (including insertion, deletion, and substitution) to enhance the activity, half-life or production of the polypeptides encoded thereby are also encompassed in the invention.

Another aspect of the present invention provides a novel method for producing BMP-2 and BMP-4 proteins. The method of the present invention involves culturing a suitable cell line, which has been transformed with a DNA sequence coding on expression for a BMP-2 or BMP-4 protein, under the control of known regulatory sequences. The transformed host cells are cultured and the BMP-2 or BMP-4 proteins recovered and purified from the culture medium. The purified proteins are substantially free from other proteins with which they are co-produced as well as from other contaminants. Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in

the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U.S. Patent 4,419,446. Another suitable mammalian cell line, which is described in the accompanying examples, is the monkey COS-1 cell line. The mammalian cell CV-1 may also be suitable.

Bacterial cells may also be suitable hosts. For example, the various strains of E. coli (e.g., HB101, MC1061) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art may also be available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

Another aspect of the present invention provides vectors for use in expression of these novel BMP-2 and BMP-4 polypeptides. Preferably the vectors contain the full novel DNA sequences described above which encode the novel factors of the invention. Additionally the vectors also contain appropriate expression control sequences permitting expression of the BMP-2 and BMP-4 protein sequences. Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present invention and useful in the production of the BMP-2 and BMP-4 proteins. The vectors may be employed in the method of transforming cell lines and contain selected regulatory sequences in operative association with the DNA coding sequences of the invention which are capable of directing the replication and expression thereof in selected host cells. Useful regulatory sequences for such vectors are known to one of skill in the art and may be selected depending upon the selected host cells. Such

selection is routine and does not form part of the present invention.

A protein of the present invention, which induces cartilage and/or bone growth in circumstances where bone is not normally formed, has application in the healing of bone fractures and cartilage defects in humans and other animals. Such a preparation employing a BMP-2 or BMP-4 protein may have prophylactic use in closed as well as open fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery. A BMP-2 or BMP-4 protein may be used in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. BMP-2 and BMP-4 polypeptides of the invention may also be useful in the treatment of osteoporosis. A variety of osteogenic, cartilage-inducing and bone inducing factors have been described. See, e.g. European patent applications 148,155 and 169,016 for discussions thereof.

The proteins of the invention may also be used in wound healing and related tissue repair. The types of wounds include, but are not limited to burns, incisions and ulcers. (See, e.g. PCT Publication WO84/01106 for discussion of wound healing and related tissue repair).

The proteins of the invention may increase neuronal survival and therefore be useful in transplantation and treatment of conditions exhibiting a decrease in neuronal survival.

A further aspect of the invention is a therapeutic method and composition for repairing fractures and other conditions related to cartilage and/or bone defects or periodontal diseases. In addition, the invention comprises therapeutic

methods and compositions for wound healing and tissue repair. Such compositions comprise a therapeutically effective amount of a BMP-2 or BMP-4 protein of the invention in admixture with a pharmaceutically acceptable vehicle, carrier or matrix.

It is expected that the proteins of the invention may act in concert with or perhaps synergistically with other related proteins and growth factors. Further therapeutic methods and compositions of the invention therefore comprise a therapeutic amount of a BMP-2 or BMP-4 protein of the invention with a therapeutic amount of at least one of the other BMP proteins disclosed in co-owned and concurrently filed U.S. applications described above. Such combinations may comprise separate molecules of the BMP proteins or heteromolecules comprised of different BMP moieties. For example, a BMP-2 or BMP-4 subunit may be linked to a BMP-1, BMP-3, BMP-5, BMP-6, BMP-7 or BMP-8 subunit. Such linkage may comprise disulfide bonds. A method and composition of the invention may comprise a disulfide linked dimer comprising a BMP-2 or BMP-4 protein subunit and another "BMP" protein subunit described above. One may comprise a heterodimer of BMP-2 and BMP-4 moieties. Another embodiment may comprise a heterodimer of BMP-2 and BMP-7 subunits.

In further compositions, BMP-2 and BMP-4 proteins may be combined with other agents beneficial to the treatment of the bone and/or cartilage defect, wound, or tissue in question. These agents include various growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factors (TGF-a and TGF-b), and insulin-like growth factor (IGF). The preparation and formulation of such physiologically acceptable protein compositions, having due regard to pH, isotonicity, stability and the like, is within the skill of the art. The therapeutic compositions are also presently valuable for veterinary applications due to the lack of species specificity in BMP proteins. Particularly domestic animals and thoroughbred horses in addition to humans

are desired patients for such treatment with BMP-2 and BMP-4 of the present invention.

BMP-2 may be used individually in a pharmaceutical composition. BMP-2 may also be used in combination with BMP-4 and/or one or more of the other BMP proteins disclosed in co-owned and co-pending US applications as discussed above. BMP-4 may be used individually in pharmaceutical composition. In addition, it may be used in combination with other BMP proteins as described above.

The therapeutic method includes administering the composition topically, systematically, or locally as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone, cartilage or tissue damage. Topical administration may be suitable for wound healing and tissue repair. Therapeutically useful agents other than the BMP-2 and BMP-4 proteins which may also optionally be included in the composition as described above, may alternatively or additionally, be administered simultaneously or sequentially with the BMP composition in the methods of the invention. Preferably for bone and/or cartilage formation, the composition would include a matrix capable of delivering BMP-2, BMP-4 or other BMP proteins to the site of bone and/or cartilage damage, providing a structure for the developing bone and cartilage and optimally capable of being resorbed into the body. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the BMP-2 and BMP-4 compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined

calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid, polyglycolic acid and polyanhydrides. Other potential materials are biodegradable and biologically well defined, such as bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate and processing to alter pore size, particle size, particle shape, and biodegradability.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of the BMP-2 and BMP-4 proteins, e.g. amount of bone weight desired to be formed, the site of bone damage, the condition of the damaged bone, the size of a wound, type of damaged tissue, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the types of BMP proteins in the composition. The addition of other known growth factors, such as IGF I (insulin like growth factor I), to the final composition, may also effect the dosage. Progress can be monitored by periodic assessment of bone growth and/or repair, for example, x-rays, histomorphometric determinations and tetracycline labeling.

The following examples illustrate practice of the present invention in recovering and characterizing bovine BMP-2 protein and employing it to recover the human proteins BMP-2 and BMP-4, and in expressing the proteins via recombinant techniques.

EXAMPLE I

Isolation of Bovine Bone Inductive Factor

Ground bovine bone powder (20-120 mesh, Helitrex) is prepared according to the procedures of M. R. Urist et al., Proc. Natl Acad. Sci USA, 70:3511 (1973) with elimination of some extraction steps as identified below. Ten kgs of the ground powder is demineralized in successive changes of 0.6N HCl at 4°C over a 48 hour period with vigorous stirring. The resulting suspension is extracted for 16 hours at 4°C with 50 liters of 2M $CaCl_2$ and 10mM ethylenediamine-tetraacetic acid [EDTA], and followed by extraction for 4 hours in 50 liters of 0.5M EDTA. The residue is washed three times with distilled water before its resuspension in 20 liters of 4M guanidine hydrochloride [GuCl], 20mM Tris (pH 7.4), 1mM N-ethylmaleimide, 1mM iodoacetamide, 1mM phenylmethylsulfonyl fluorine as described in Clin. Orthop. Rel. Res., 171: 213 (1982). After 16 to 20 hours the supernatant is removed and replaced with another 10 liters of GuCl buffer. The residue is extracted for another 24 hours.

The crude GuCl extracts are combined, concentrated approximately 20 times on a Pellicon apparatus with a 10,000 molecular weight cut-off membrane, and then dialyzed in 50mM Tris, 0.1M NaCl, 6M urea (pH7.2), the starting buffer for the first column. After extensive dialysis the protein is loaded on a 4 liter DEAE cellulose column and the unbound fractions are collected.

The unbound fractions are concentrated and dialyzed against 50mM NaAc, 50mM NaCl (pH 4.6) in 6M urea. The unbound fractions are applied to a carboxymethyl cellulose column. Protein not bound to the column is removed by extensive washing with starting buffer, and the material containing protein having bone and/or cartilage formation activity as measured by the Rosen-modified Sampath - Reddi assay (described in Example III below) desorbed from the column by 50mM NaAc, 0.25mM NaCl, 6M urea (pH 4.6). The protein from this step elution is con-

centrated 20- to 40- fold, then diluted 5 times with 80mM KPO_4, 6M urea (pH 6.0). The pH of the solution is adjusted to 6.0 with 500mM K_2HPO_4. The sample is applied to an hydroxylapatite column (LKB) equilibrated in 80mM KPO_4, 6M urea (pH 6.0) and all unbound protein is removed by washing the column with the same buffer. Protein having bone and/or cartilage formation activity is eluted with 100mM KPO_4 (pH7.4) and 6M urea.

The protein is concentrated approximately 10 times, and solid NaCl added to a final concentration of 0.15M. This material is applied to a heparin - Sepharose column equilibrated in 50mM KPO_4, 150mM NaCl, 6M urea (pH 7.4). After extensive washing of the column with starting buffer, a protein with bone and/or cartilage inductive activity is eluted by 50mM KPO_4, 700mM NaCl, 6M urea (pH 7.4). This fraction is concentrated to a minimum volume, and 0.4ml aliquots are applied to Superose 6 and Superose 12 columns connected in series, equilibrated with 4M GuCl, 20mM Tris (pH 7.2) and the columns developed at a flow rate of 0.25ml/min. The protein demonstrating bone and/or cartilage inductive activity has a relative migration on SDS-PAGE corresponding to approximately 30,000 dalton protein.

The above fractions from the superose columns are pooled, dialyzed against 50mM NaAc, 6M urea (pH 4.6), and applied to a Pharmacia MonoS HR column. The column is developed with a gradient to 1.0M NaCl, 50mM NaAc, 6M urea (pH 4.6). Active bone and/or cartilage formation fractions are pooled and brought to pH 3.0 with 10% trifluoroacetic acid (TFA). The material is applied to a 0.46 x 25cm Vydac C4 column in 0.1% TFA and the column developed with a gradient to 90% acetonitrile, 0.1% TFA (31.5% acetonitrile, 0.1% TFA to 49.5% acetonitrile, 0.1% TFA in 60 minutes at 1ml per minute). Active material is eluted at approximately 40-44% acetonitrile. Aliquots of the appropriate active fractions are iodinated by one of the following methods: P. J. McConahey et

al, Int. Arch. Allergy, 29:185-189 (1966); A. E. Bolton et al, Biochem J., 133:529 (1973); and D. F. Bowen-Pope, J. Biol. Chem., 237:5161 (1982). The iodinated proteins present in these fractions are analyzed by SDS gel electrophoresis and urea Triton X 100 isoelectric focusing. At this stage, the protein having bone and/or cartilage forming activity is estimated to be approximately 10-50% pure.

EXAMPLE II

Characterization of Bovine Bone Inductive Factor

A. Molecular Weight

Approximately 20ug protein from Example I is lyophilized and redissolved in 1X SDS sample buffer. After 15 minutes of heating at 37°C, the sample is applied to a 15% SDS polyacrylamide gel and then electrophoresed with cooling. The molecular weight is determined relative to prestained molecular weight standards (Bethesda Research Labs). Immediately after completion, the gel lane containing bone and/or cartilage forming material is sliced into 0.3cm pieces. Each piece is mashed and 1.4ml of 0.1% SDS is added. The samples are shaken gently overnight at room temperature to elute the protein. Each gel slice is desalted to prevent interference in the biological assay. The supernatant from each sample is acidified to pH 3.0 with 10% TFA, filtered through a 0.45 micron membrane and loaded on a 0.46cm x 5cm C4 Vydac column developed with a gradient of 0.1% TFA to 0.1% TFA, 90% CH_3CN. The appropriate bone and/or cartilage inductive protein - containing fractions are pooled and reconstituted with 20mg rat matrix and assayed. In this gel system, the majority of bone and/or cartilage inductive fractions have the mobility of a protein having a molecular weight of approximately 28,000 - 30,000 daltons.

B. Isoelectric Focusing

The isoelectric point of bone inductive factor activity

is determined in a denaturing isoelectric focusing system. The Triton X100 urea gel system (Hoeffer Scientific) is modified as follows: 1) 40% of the ampholytes used are Servalyte 3/10; 60% are Servalyte 7-9; and 2) the catholyte used is 40mM NaOH. Approximately 20ug of protein from Example I is lyophilized, dissolved in sample buffer and applied to the isoelectrofocusing gel. The gel is run at 20 watts, 10 C for approximately 3 hours. At completion the lane containing bone and/or cartilage inductive factor is sliced into 0.5 cm slices. Each piece is mashed in 1.0ml 6M urea, 5mM Tris (pH 7.8) and the samples agitated at room temperature. The samples are acidified, filtered, desalted and assayed as described above. The major portion of activity as determined by the Rosen-modified Sampath - Reddi assay migrates in a manner consistent with a pI of about 8.8 - 9.2.

C. Subunit Characterization

The subunit composition of the isolated bovine bone protein is also determined. Pure bone inductive factor is isolated from a preparative 15% SDS gel as described above. A portion of the sample is then reduced with 5mM DTT in sample buffer and re-electrophoresed on a 15% SDS gel. The approximately 28-30kd protein yields two major bands at approximately 18-20kd and approximately 16-18kd, as well as a minor band at approximately 28-30kd. The broadness of the two bands indicates heterogeneity caused most probably by glycosylation, other post translational modification, proteolytic degradation or carbamylation.

EXAMPLE III

Rosen Modified Sampath-Reddi Assay

A modified version of the rat bone formation assay described in Sampath and Reddi, Proc. Natl. Acad. Sci. U.S.A., 80:6591-6595 (1983) is used to evaluate bone and/or cartilage activity of the bovine protein obtained in Example I and the

BMP-2 proteins of the invention. This modified assay is herein called the Rosen-modified Sampath-Reddi assay. The ethanol precipitation step of the Sampath-Reddi procedure is replaced by dialyzing (if the composition is a solution) or diafiltering (if the composition is a suspension) the fraction to be assayed against water. The solution or suspension is then redissolved in 0.1 % TFA, and the resulting solution added to 20mg of rat matrix. A mock rat matrix sample not treated with the protein serves as a control. This material is frozen and lyophilized and the resulting powder enclosed in #5 gelatin capsules. The capsules are implanted subcutaneously in the abdominal thoracic area of 21 - 49 day old male Long Evans rats. The implants are removed after 7 - 14 days. Half of each implant is used for alkaline phosphatase analysis [See, A. H. Reddi et al., Proc. Natl Acad Sci., 69:1601 (1972)].

The other half of each implant is fixed and processed for histological analysis. 1μm glycolmethacrylate sections are stained with Von Kossa and acid fuschin to score the amount of induced bone and cartilage formation present in each implant. The terms +1 through +5 represent the area of each histological section of an implant occupied by new bone and/or cartilage cells and matrix. A score of +5 indicates that greater than 50% of the implant is new bone and/or cartilage produced as a direct result of protein in the implant. A score of +4, +3, +2 and +1 would indicate that greater than 40%, 30%, 20% and 10% respectively of the implant contains new cartilage and/or bone.

The rat matrix samples containing at least 200 ng of bovine protein obtained in Example I result in bone and/or cartilage formation that filled more than 20% of the implant areas sectioned for histology. This protein therefore scores at least +2 in the Rosen-modified Sampath-Reddi assay. The dose response of the matrix samples indicates that the amount of bone and/or cartilage formed increases with the amount of protein in the sample. The control sample did not result in

any bone and/or cartilage formation. The purity of the protein assayed is approximately 10-15% pure.

The bone and/or cartilage formed is physically confined to the space occupied by the matrix. Samples are also analyzed by SDS gel electrophoresis and isoelectric focusing as described above, followed by autoradiography. Analysis reveals a correlation of activity with protein bands at 28 - 30kd and a pI of approximately 8.8 - 9.2. To estimate the purity of the protein in a particular fraction an extinction coefficient of 1 OD/mg-cm is used as an estimate for protein and the protein is run on SDS PAGE followed by silver staining or radioiodination and autoradiography.

EXAMPLE IV

<u>Bovine BMP-2</u>

The protein composition of Example IIA of molecular weight 28 - 30kd is reduced as described in Example IIC and digested with trypsin. Eight tryptic fragments are isolated by standard procedures having the following amino acid sequences:

Fragment 1: A A F L G D I A L D E E D L G
Fragment 2: A F Q V Q Q A A D L
Fragment 3: N Y Q D M V V E G
Fragment 4: S T P A Q D V S R
Fragment 5: N Q E A L R
Fragment 6: L S E P D P S H T L E E
Fragment 7: F D A Y Y
Fragment 8: L K P S N ? A T I Q S I V E

Two probes consisting of pools of oligonucleotides (or unique oligonucleotides) are designed according to the method of R. Lathe, <u>J. Mol. Biol.</u>, 183(1):1-12 (1985) on the basis of the amino acid sequence of Fragment 3 and synthesized on an automated DNA synthesizer as described above.

Probe #1: A C N A C C A T [A/G] T C [T/C] T G [A/G] A T

Probe #2: C A [A/G] G A [T/C] A T G G T N G T N G A

Because the genetic code is degenerate (more than one codon can code for the same amino acid), the number of oligonucleotides in a probe pool is reduced based on the frequency of codon usage in eukaryotes, the relative stability of G:T base pairs, and the relative infrequency of the dinucleotide CpG in eukaryotic coding sequences [See J. J. Toole et al, Nature, 312:342-347 (1984)]. Bracketed nucleotides are alternatives. "N" means either A, T, C or G. These probes are radioactively labeled and employed to screen a bovine genomic library. The library is constructed as follows: Bovine liver DNA is partially digested with the restriction endonuclease enzyme Sau 3A and sedimented through a sucrose gradient. Size fractionated DNA in the range of 15-30kb is then ligated to the vector lambda J' Bam H1 arms [Mullins et al., Nature, 308:856-858 (1984)]. The library is plated at 8000 recombinants per plate. Duplicate nitrocellulose replicas of the plaques are made and amplified according to a modification of the procedure of Woo et al, Proc. Natl. Acad. Sci. USA, 75:3688-91 (1978). Probe #1 is hybridized to the set of filters in 3M tetramethylammonium chloride (TMAC), 0.1M sodium phosphate pH6.5, 1mM EDTA, 5X Denhardts, 0.6% SDS, 100ug/ml salmon sperm DNA at 48 degrees C, and washed in 3M TMAC, 50mM Tris pH 8.0 at 50 degrees C. These conditions minimize the detection of mismatches to the 17 mer probe pool [see, Wood et al, Proc. Natl. Acad. Sci, U.S.A., 82:1585-1588 (1985)].

400,000 recombinants are screened by this procedure. One duplicate positive is plaque purified and the DNA is isolated from a plate lysate of the recombinant bacteriophage designated lambda bP-21. Bacteriophage bP-21 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland USA (hereinafter the "ATCC") under accession number ATCC 40310 on March 6, 1987. This deposit as well as the other deposits contained herein meets the requirements of the Budapest Treaty on the International

Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and Regulations thereunder. The bP-21 clone encodes at least a portion of a bovine BMP-2 protein designated bovine BMP-2 or bBMP-2.

The oligonucleotide hybridizing region of this BMP-2 clone is localized to an approximately 1.2 kb Sac I restriction fragment which is subcloned into M13 and sequenced by standard techniques. The partial DNA sequence and derived amino acid sequence of this Sac I fragment and the contiguous Hind III - Sac I restriction fragment of bP-21 are shown below in Figure 1 (SEQ ID NO: 1). The BMP-2 peptide sequence from this clone is 129 amino acids in length and is encoded by the DNA sequence from nucleotide #1 through nucleotide #387 (SEQ ID NO:1). The amino acid sequence corresponding to the tryptic fragment isolated from the bovine bone 28 to 30kd material is underlined in Figure 1. The underlined portion of the sequence corresponds to tryptic Fragment 3 above from which the oligonucleotide probes for BMP-2 are designed. The predicted amino acid sequence indicates that tryptic Fragment 3 is preceded by a basic residue (K) as expected considering the specificity of trypsin. The arginine residue encoded by the CGT triplet is presumed to be the carboxy-terminus of the protein based on the presence of a stop codon (TAG) adjacent to it.

EXAMPLE V

Human BMP-2 and BMP-4

The HindIII-SacI bovine genomic BMP-2 fragment described in Example IV is subcloned into an M13 vector. A ^{32}P-labeled single-stranded DNA probe is made from a template preparation of this subclone. This probe is used to screen polyadenylated RNAs from various cell and tissue sources. Polyadenylated RNAs from various cell and tissue sources are electrophoresed on formaldehyde-agarose gels and transferred to nitrocellulose by the method of Toole et al., supra. The probe is then hybridized to the nitrocellulose blot in 50% formamide, 5 X SSC, 0.1% SDS, 40 mM sodium phosphate pH 6.5, 100 ug/ml denatured salmon sperm DNA, and 5 mM vanadyl ribonucleosides at 42° C overnight and washed at 65° C in 0.2 X SSC, 0.1% SDS. A hybridizing band corresponding to an mRNA species of approximately 3.8 kb is detected in the lane containing RNA from the human osteosarcoma cell line U-2 OS. cDNA is synthesized from U-2 OS polyadenylated RNA and cloned into lambda GT10 by established techniques (Toole et al, supra). 20,000 recombinants from this library are plated on each of 50 plates. Duplicate nitrocellolose replicas are made of the plates.

The HindIII-SacI fragment is labeled with ^{32}P by nick translation and used to screen the nitrocellulose filter replicas of the above-described U-2 OS cDNA library by hybridization in standard hybridization buffer at 65° overnight followed by washing in 1 X SSC, 0.1% SDS at 65°. Twelve duplicate positive clones are picked and replated for secondaries. Duplicate nitrocellulose replicas are made of the secondary plates and both sets hybridized to the bovine genomic probe as the primary screening was performed. One set of filters is then washed in 1 X SSC, 0.1% SDS; the other in 0.1 X SSC, 0.1% SDS at 65°.

Two classes of hBMP-2 cDNA clones are evident based on strong (4 recombinants) or weak (7 recombinants) hybridization

signals under the more stringent washing conditions (0.1 X SSC, 0.1% SDS). All 11 recombinant bacteriophage are plaque purified, small scale DNA preparations made from plate lysates of each, and the inserts subcloned into pSP65 and into M13 for sequence analysis. Sequence analysis of the strongly hybridizing clones designated hBMP-2 (previously designated BMP-2A and BMP-2 Class I) indicates that they have extensive sequence homology with the sequence given in Figure 1 (SEQ ID NO: 1). These clones are therefore cDNA encoding the human equivalent of the protein encoded by the bBMP-2 gene whose partial sequence is given in Figure 1. Sequence analysis of the weakly hybridizing recombinants designated hBMP-4 (previously designated BMP-2B and BMP-2 Class II) indicates that they are also quite homologous with the sequence given in Figure 1 (SEQ ID NO: 1) at the 3' end of their coding regions, but less so in the more 5' regions. Thus they encode a human protein of similar, though not identical, structure to that above.

Full length human BMP-2 cDNA clones are obtained in the following manner. The 1.5 kb insert of one of the BMP-4 subclones (II-10-1) is isolated and radioactively labeled by nick-translation. One set of the nitrocellulose replicas of the U-2 OS cDNA library screened above (50 filters, corresponding to 1,000,000 recombinant bacteriophage) are rehybridized with this probe under stringent conditions (hybridization at 65° in standard hybridization buffer; washing at 65° in 0.2 X SSC, 0.1% SDS). All recombinants which hybridize to the bovine genomic probe which do not hybridize to the BMP-4 probe are picked and plaque purified (10 recombinants). Plate stocks are made and small scale bacteriophage DNA preparations made. After subcloning into M13, sequence analysis indicates that 4 of these represent clones which overlap the original BMP-2 clone. One of these, lambda U2OS-39, contains an approximately 1.5 kb insert and was deposited with the ATCC on June 16, 1987 under accession

number 40345. The DNA sequence (SEQ ID NO: 3) (compiled from lambda U20S-39 and several other hBMP-2 cDNA recombinants) and derived amino acid sequence (SEQ ID NO: 4) are shown below in Figure 2. Lambda U20S-39 is expected to contain all of the nucleotide sequence necessary to encode the entire human counterpart of the protein BMP-2 encoded by the bovine gene segment whose partial sequence is presented in Figure 1. The BMP-2 protein encoded by the DNA sequence of Figure 2 is contemplated to contain the 97 amino acid sequence from amino acid #299 to #396 or a sequence substantially homologous thereto. This human cDNA hBMP-2 contains an open reading frame of 1188 bp, encoding a protein of 396 amino acids. The protein is preceded by a 5' untranslated region of 342 bp with stop codons in all frames. The 13 bp region preceding this 5' untranslated region represents a linker used in the cDNA cloning procedure. This protein of 396 amino acids has a molecular weight of 45kd based on this amino acid sequence. It is contemplated that this sequence represents the primary translation product. It is further contemplated that BMP-2 may correspond to the approximately 18 - 20kd subunit of Example IIC. The sequence corresponding to the sequence tryptic Fragment 3 of Example IV is underlined in Figure 2. The "pre" portion of the human BMP-2 protein is contemplated to comprise amino acid #1 to amino acid #23 as shown in Figure 2. The "pro" portion is contemplated to comprise amino acid #24 to amino acid #282 of Figure 2 (SEQ ID NO: 4). The mature portion is contemplated to comprise amino acid #283 (Gln, Ala, Lys...) to #396 (Arg) of Figure 2.

BMP-2 proteins of the invention comprise at least the amino acid sequence from amino acid #299 to #396, although further included in the invention are protein species with a carboxy terminus which is characterized by an amino acid upstream from amino acid #396.

Full-length BMP-4 human cDNA clones are obtained in the following manner. The 200 bp EcoRI-SacI fragment from the 5'

end of the BMP-4 recombinant II-10-1 is isolated from its plasmid subclone, labeled by nick-translation, and hybridized to a set of duplicate nitrocellulose replicas of the U-2 OS cDNA library (25 filters/set; representing 500,000 recombinants). Hybridization and washing are performed under stringent conditions as described above. 16 duplicate positives are picked and replated for secondaries. Nitrocellulose filter replicas of the secondary plates are made and hybridized to an oligonucleotide which was synthesized to correspond to the sequence of II-10-1 and is of the following sequence:

CGGGCGCTCAGGATACTCAAGACCAGTGCTG

Hybridization is in standard hybridization buffer AT 50° C with washing at 50° in 1 X SSC, 0.1% SDS. 14 recombinant bacteriophage which hybridize to this oligonucleotide are plaque purified. Plate stocks are made and small scale bacteriophage DNA preparations made. After sucloning 3 of these into M13, sequence analysis indicates that they represent clones which overlap the original BMP-4 clone. One of these, lambda U2OS-3, was deposited with the ATCC under accession number 40342 on June 16, 1987. U2OS-3 contains an insert of approximately 1.8 kb. The DNA sequence (SEQ ID NO:5) and derived amino acid sequence (SEQ ID NO: 6) of U2OS-3 are shown below in Figure 3. This clone is expected to contain all of the nucleotide sequence necessary to encode the entire human BMP-4 protein. The BMP-4 protein encoded by Figure 3 is contemplated to contain the 97 amino acid sequence from amino acid #311 to #408 or a sequence substantially homologous thereto. This cDNA contains an open reading frame of 1224 bp, encoding a protein of 408 amino acids, preceded by a 5' untranslated region of 394 bp with stop codons in all frames, and contains a 3' untranslated region of 308 bp following the in-frame stop codon. The 8 bp region preceding the 5' untranslated region represents a linker used in the cDNA cloning procedure. This protein of 408 amino acids has

molecular weight of 47kd and is contemplated to represent the primary translation product. Mature BMP-4 is contemplated to comprise amino acid #293(Ser, Pro, Lys...) - #408 (Arg) of Figure 3. A sequence similar though not idential to tryptic Fragment 3 of Example IV is underlined in Figure 3 (SEQ ID NO: 6). The underlined sequence Asn-Tyr-Gln-Glu-Met-Val-Val-Glu-Gly differs from the tryptic fragment Asn-Tyr-Gln-Asp-Met-Val-Val-Glu-Gly by one amino acid in position four.

The sequences of BMP-2 and BMP-4, as shown in Figures 2 and 3, have significant homology to the beta (B) and beta (A) subunits of the inhibins. The inhibins are a family of hormones which are presently being investigated for use in contraception. See, A. J. Mason et al, Nature, 318:659-663 (1985). To a lesser extent they are also homologous to Mullerian inhibiting substance (MIS), a testicular glycoprotein that causes regression of the Mullerian duct during development of the male embryo, and transforming growth factor-beta (TGF-β) which can inhibit or stimulate growth of cells or cause them to differentiate. Furthermore, the sequences of Figures 2 and 3 indicate that BMP-2 and BMP-4 have significant homology to the Drosophila decapentaplegic (DPP-C) locus transcript. See, J. Massague, Cell, 49:437-438 (1987); R. W. Padgett et al, Nature, 325:81-84 (1987); R. L. Cate et al, Cell 45: 685-698 (1986). It is considered possible therefore that a BMP-2 protein is the human homolog of the protein made from this transcript from this developmental mutant locus. BMP-2 and BMP-4 share sequence similarity with Vgl. Vgl mRNA has been localized to the vegetal hemisphere of Xenopus oocytes. During early development, it is distributed throughout the endoderm, but the mRNA is not detectable after blastula formation has occurred. The Vgl protein may be the signal used by the endoderm cells to commit ectodermal cells to become the embryonic mesoderm.

The procedures described above may be employed to isolate other related BMP-2 and BMP-4 proteins of interest by utilizing

the bovine BMP-2 and BMP-4 proteins as a probe source. Such other BMP-2 and BMP-4 proteins may find similar utility in, inter alia, fracture repair, wound healing and tissue repair.

EXAMPLE VI

Expression of BMP-2 and BMP-4

In order to produce bovine, human or other mammalian BMP-2 and BMP-4 proteins, the DNA encoding the desired protein is transferred into an appropriate expression vector and introduced into mammalian cells or other preferred eukaryotic or prokaryotic hosts by conventional genetic engineering techniques. The presently preferred expression system for biologically active recombinant human BMP-2 and BMP-4 is stably transformed mammalian cells.

One skilled in the art can construct mammalian expression vectors by employing the sequence of Figures 1 - 3 (SEQ ID NO: 1,3, and 5), or other DNA sequences containing the coding sequences of Figures 1-3, or other modified sequences and known vectors, such as pCD [Okayama et al., Mol. Cell Biol., 2:161-170 (1982)] and pJL3, pJL4 [Gough et al., EMBO J., 4:645-653 (1985)]. The BMP-2 and BMP-4 cDNA sequences can be modified by removing the non-coding nucleotides on the 5' and 3' ends of the coding region. The deleted non-coding nucleotides may or may not be replaced by other sequences known to be beneficial for expression. The transformation of these vectors into appropriate host cells can result in expression of BMP-2 or BMP-4 proteins.

One skilled in the art could manipulate the sequences of Figures 1-3 (SEQ ID NO: 1,3, and 5) by eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression by bacterial cells. For example, the coding sequences could be further manipulated (e.g. ligated to other known linkers or modified by deleting

non-coding sequences there-from or altering nucleotides therein by other known techniques). The modified BMP-2 or BMP-4 coding sequence could then be inserted into a known bacterial vector using procedures such as described in T. Taniguchi et al., Proc. Natl Acad. Sci. USA, 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells and BMP-2 protein or BMP-4 expressed thereby. For a strategy for producing extracellular expression of BMP-2 or BMP-4 proteins in bacterial cells., see, e.g. European patent application EPA 177,343.

Similar manipulations can be performed for the construction of an insect vector [See, e.g. procedures described in published European patent application 155,476] for expression in insect cells. A yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the factors of the present invention by yeast cells. [See, e.g., procedures described in published PCT application WO86/00639 and European patent application EPA 123,289].

A method for producing high levels of a BMP-2 or BMP-4 protein of the invention in mammalian cells involves the construction of cells containing multiple copies of the heterologous BMP-2 or BMP-4 gene. The heterologous gene is linked to an amplifiable marker, e.g. the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol., 159:601-629 (1982). This approach can be employed with a number of different cell types.

For example, a plasmid containing a DNA sequence for a BMP-2 or BMP-4 of the invention in operative association with other plasmid sequences enabling expression thereof and the DHFR expression plasmid pAdA26SV(A)3 [Kaufman and Sharp, Mol. Cell. Biol., 2:1304 (1982)] can be co-introduced into DHFR-

deficient CHO cells, DUKX-BII, by calcium phosphate coprecipitation and transfection, electroperation, protoplast fusion or lipofection. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of MTX (e.g. sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as described in Kaufman et al., Mol Cell Biol., 5:1750 (1983).

Transformants are cloned, and biologically active BMP-2 or BMP-4 expression is monitored by the Rosen-modified Sampath - Reddi rat bone formation assay described above in Example III. BMP-2 and BMP-4 expression should increase with increasing levels of MTX resistance. Similar procedures can be followed to produce other related BMP-2 and BMP-4 proteins.

A. COS Cell Expression

As one specific example of producing a BMP-2 protein of the invention, the insert of II-3 (a λ GT10 derivative containing the full length BMP-2 cDNA) is released from the vector arms by digestion with EcoRI and subcloned into pSP65 (Promega Biotec, Madison, Wisconsin)[Melton et al, Nucl.Acids Res. 12:7035-7056 (1984)] in both orientations yielding pBMP-2 #39-3 or pBMP-2 #39-4. The insert is subcloned into the EcoRI site of the mammalian expression vector, pMT2 CXM, described below, though derivitives thereof may also be suitable. Plasmid DNA from this subclone is transfected into COS cells by the DEAE-dextran procedure [Sompayrac and Danna PNAS 78:7575-7578 (1981); Luthman and Magnusson, Nucl.Acids Res. 11: 1295-1308 (1983)] and the cells are cultured. Serum-free 24 hr. conditioned medium is collected from the cells starting 40 - 70 hr. post-transfection. Recovery and purification of the COS expressed BMP-2 proteins is described below in Example VII.

The mammalian expression vector pMT2 CXM is a derivative of p91023 (b) (Wong et al., Science 228:810-815, 1985)

differing from the latter in that it contains the ampicillin resistance gene in place of the tetracycline resistance gene and further contains a XhoI site for insertion of cDNA clones. The functional elements of pMT2 CXM have been described (Kaufman, R.J., 1985, Proc. Natl. Acad. Sci. USA 82:689-693) and include the adenovirus VA genes, the SV40 origin of replication including the 72 bp enhancer, the adenovirus major late promoter including a 5' splice site and the majority of the adenovirus tripartite leader sequence present on adenovirus late mRNAs, a 3' splice acceptor site, a DHFR insert, the SV40 early polyadenylation site (SV40), and pBR322 sequences needed for propagation in <u>E. coli</u>.

Plasmid pMT2 CXM is obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC 67122. EcoRI digestion excises the cDNA insert present in pMT2-VWF, yielding pMT2 in linear form which can be ligated and used to transform <u>E</u>. <u>coli</u> HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods. pMT2 CXM is then constructed using loopout/in mutagenesis [Morinaga, et al., <u>Biotechnology</u> <u>84</u>: 636 (1984). This removes bases 1075 to 1145 relative to the Hind III site near the SV40 origin of replication and enhancer sequences of pMT2. In addition it inserts the following sequence:

5' PO$_{-C}$ATGGGCAGCTCGAG-3'

at nucleotide 1145. This sequence contains the recognition site for the restriction endonuclease Xho I. A derivative of pMT2CXM, termed pMT23, contains recognition sites for the restriction endonucleases PstI, Eco RI, SalI and XhoI. Plasmid pMT2 CXM and pMT23 DNA may be prepared by conventional methods.

B. CHO Cell Expression

(1) <u>BMP-2 Expression in CHO Cells</u>

In order to achieve high levels of human BMP-2 protein expression, the DNA sequence of Figure 2 (SEQ ID NO:3) encoding

BMP-2 is inserted into a eucaryotic expression vector, stably introduced into CHO cells and amplified to high copy number by methotrexate selection of DHFR [R.J. Kaufman, et al., EMBO J. 6:189 (1987)]. The transformed cells are cultured and the expressed BMP-2 proteins are recovered and purified from the culture media.

A BMP-2 protein of the invention is expressed in CHO cells by releasing the insert of pBMP-2 #39-3 described above, from the vector by digestion with EcoRI. The insert is subcloned into the EcoRI cloning site of the mammalian expression vector, pMT2 CXM described above, though derivitives thereof may also be suitable.

A derivative of the BMP-2 cDNA sequence set forth in Figure 2 (SEQ ID NO: 3) in which the 5' untranslated region is deleted is made by removal of the sequences contained between the SalI site at the 5' adapter (from the original cDNA cloning), and the SalI site 7 base pairs upstream of the initiator ATG, by digestion with SalI and religation. This step is conveniently performed in either SP65 derivatives containing the full length BMP-2 cDNA, but can also be performed in pMT2 derivatives. The 3' untranslated region is removed using heteroduplex mutagenesis using the mutagenic oligonucleotide

5' GAGGGTTGTGGGTGTCGCTAGTGAGTCGACTACAGCAAAATT
Terminator SalI

The sequence contains the terminal 3' coding region of the BMP-2 cDNA, followed immediately by a recognition site for SalI.

The BMP-2 cDNA with deletions of the 5' and 3' untranslated regions are excised from pSP65 with SalI, and subcloned into the SalI site of pMT23 described above. Plasmid DNA from the subclones is transfected into CHO cells by electroporation [Neuman et al, EMBO J., 1:841-845 (1982)]. Two days later, cells are switched to selective medium containing 10% dialyzed fetal bovine serum and lacking nucleosides. Colonies expressing DHFR are counted 10-14 days later.

Individual colonies or pools of colonies are expanded and analyzed for expression of BMP-2 RNA and protein using standard procedures and are subsequently selected for amplification by growth in increasing concentrations of MTX. Stepwise selection of the preferred pool, termed 2ΔD, is carried out up to a concentration of 2 μM MTX. Individual cells from the pool are then cloned and assayed for BMP-2 expression. Procedures for such assay include Northern Blot analysis to detect the presence of mRNA, protein analysis including SDS-PAGE and analysis for cartilage and/or bone formation activity using the ectopic rat bone formation assay described above. The presently preferred clonally-derived cell line is identified as 2ΔD2I. This cell line secretes BMP-2 proteins into the media containing 2μM MTX.

The CHO cell line 2ΔD2I is grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12, 1:1 (vol/vol), supplemented with 10% fetal bovine serum. When the cells are 80 - 100 % confluent, the medium is replaced with serum-free DMEM/F-12. Medium is harvested every 24 hours for 4 days. For protein production and purification the cells are cultured serum-free.

Currently, this cell line 2ΔD2I is being subjected to stepwise selection in increasing concentrations of MTX (10μM, 100μM, 1000μM) which may potentially yield cells which produce even higher levels of BMP-2 protein expression.

cDNA genes inserted into the EcoRI and/or Xho I sites are expressed as a bicistronic mRNA with DHFR in the second position. In this configuration, translation of the upstream (BMP-2) open reading frame is more efficient than the downstream (DHFR) cDNA gene [Kaufman et al, EMBO J. 6:187-193 (1987). The amount of DHFR protein expressed is nevertheless sufficient for selection of stable CHO cell lines.

Characterization of the BMP-2 polypeptides through pulse labeling with [35S] methionine or cysteine and polyacrylamide gel electrophoresis indicates that multiple molecular size

forms of BMP-2 proteins, further described below, are being expressed and secreted from the stable CHO lines.

(2) BMP-4 Expression in CHO Cells

In order to achieve high levels of human BMP-4 protein expression, the DNA sequence of Figure 3 (SEQ ID NO: 5) encoding BMP-4 is inserted into a eucaryotic expression vector, stably introduced into CHO cells and amplified to high copy number by methotrexate selection of DHFR [R.J. Kaufman, et al., EMBO J. 6:189 (1987)]. The transformed cells are cultured and the expressed BMP-4 proteins are recovered and purified from the culture media.

As described above, numerous expression vectors known in the art may be utilized in the expression of BMP proteins of the invention. The vector utilized in the following example is pEMC2β1 derived from pMT21 though other vectors may be suitable in practice of the invention.

pMT21 is derived from pMT_2 which is derived from pMT2-VWF, deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC 67122 under the provisions of the Budapest Treaty. EcoRI digestion excises the cDNA insert present in pMT-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. Coli HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods.

pMT21 was derived from pMT2 through the following two modifications. First, 76 bp of the 5' untranslated region of the DHFR cDNA including a stretch of 19 G residues from G/C tailing for cDNA cloning was deleted. In this process, a XhoI site was inserted to obtain the following sequence immediately upstream from DHFR: 5' -CTGCAGGCGAGCCTGAATTCCTCGAGCCATCATG-3'

PstI Eco RI XhoI

Second, a unique ClaI site was introduced by digestion with EcoRV and XbaI, treatment with Klenow fragment of DNA polymerase I, and ligation to a ClaI linker (CATCGATG). This

deletes a 250 bp segment from the adenovirus virus associated RNA (VAI) region but does not interfere with VAI RNA gene expression or function. pMT21 was digested with EcoRI and XhoI, and used to derive the vector pEMC2B1.

A portion of the EMCV leader was obtained from pMT2-ECAT1 [S.K. Jung, et al, J. Virol 63:1651-1660 (1989)] by digest with Eco RI and PstI, resulting in a 2752 bp fragment. This fragment was digested with TaqI yielding an Eco RI-TaqI fragment of 508 bp which was purified by electrophoresis on low melting agarose gel. A 68 bp adapter and its complementary strand were synthesized with a 5' TaqI protruding end and a 3' XhoI protruding end which has the following sequence:

```
5'-CGAGGTTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTT
   TaqI

   GAAAAACACGATTGC-3'
                 XhoI
```

This sequence matches the EMC virus leader sequence from nucleotide 763 to 827. It also changes the ATG at position 10 within the EMC virus leader to an ATT and is followed by a XhoI site. A three way ligation of the pMT21 Eco RI-XhoI fragment, the EMC virus EcoRI-TaqI fragment, and the 68 bp oligonucleotide adapter TaqI-XhoI adapter resulted in the vector pEMC2B1.

This vector contains the SV40 origin of replication and enhancer, the adenovirus major late promoter, a cDNA copy of the majority of the adenovirus tripartite leader sequence, a small hybrid intervening sequence, an SV40 polyadenylation signal and the dadenovirus VA I gene, DHFR and B-lactamase markers and an EMC sequence, in appropriate relationships to direct the high level expression of the desired cDNA in mammalian cells.

A derivative of the BMP-4 cDNA sequence set forth in Figure 3 in which the 3' untranslated region is removed is made via heteroduplex mutagenesis with the mutagenic

oligonucleotide:

5' GGATGTGGGTGCCGCTGACTCTAGAGTCGACGGAATTC 3'.
Terminator EcoRI

This deletes all of the sequences 3' to the translation terminator codon of the BMP-4 cDNA, juxtaposing this terminator codon and the vector polylinker sequences. This step is performed in an SP65 vector though may be conveniently performed in MT2 derivatives containing the BMP-4 cDNA. The 5' untranslated region is removed using the restriction endonuclease BsmI, which cleaves within the eighth codon of BMP-4 cDNA. Reconstruction of the first eight codons is accomplished by ligation to oliognucleotides:

EcoRI Initiator BsmI
5' AATTCACCATGATTCCTGGTAACCGAATGCT 3' and
3' GTGGTACTAAGGACCATTGGCTTAC 5'

These oligonucleotides form a duplex which has a BsmI complementary cohesive end capable of ligation to the BsmI restricted BMP-4 cDNA, and it has an EcoRI complementary cohesive end capable of ligation to the EcoRI restricted vector MT2. Thus the cDNA for BMP-4 with the 5' and 3' untranslated regions deleted, and retaining the entire encoding sequence is contained within an EcoRI restriction fragment of approximately 1.2kb.

The BMP-4 containing plasmid designated pXMBMP-4DUT is digested with EcoRI in order to release the BMP-4 cDNA containing insert from the vector. This insert is subcloned into the EcoRI site of the mammalian expression vector pEMC2β1 described above. Plasmid DNA from the subclones is transfected into CHO cells by electroporation [Neuman et al, EMBO J., 1:841-845 (1982)]. Two days later, cells are switched to selective medium containing 10% dialyzed fetal bovine serum and lacking nucleosides. Colonies expressing DHFR are counted 10-14 days later. Individual colonies or pools of colonies are

expanded and analyzed for expression of BMP-4 RNA and protein using standard procedures and are subsequently selected for amplification by growth in increasing concentrations of MTX. Stepwise selection of the preferred pool, termed 4ΔED, is carried out up to a concentration of 2 μM MTX. Individual cells from the pool are then cloned and assayed for BMP-4 expression. Procedures for such assay include Northern Blot analysis to detect the presence of mRNA, protein analysis including SDS-PAGE and analysis for cartilage and/or bone formation activity using the ectopic rat bone formation assay described above.

4ΔED is grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12, 1:1 (vol/vol), supplemented with 10% fetal bovine serum. When the cells are 80 - 100 % confluent, the medium is replaced with serum-free DMEM/F-12. Medium is harvested every 24 hours for 4 days. For protein production and purification the cells are cultured serum-free.

cDNA genes inserted into the EcoRI and/or Xho I sites are expressed as a bicistronic mRNA with DHFR in the second position. In this configuration, translation of the upstream (BMP-4) open reading frame is more efficient than the downstream (DHFR) cDNA gene [Kaufman et al, EMBO J. 6:187-193 (1987). The amount of DHFR protein expressed is nevertheless sufficient for selection of stable CHO cell lines.

Characterization of the BMP-4 polypeptides through pulse labeling with [35S] methionine or cysteine and polyacrylamide gel electrophoresis indicates that multiple molecular size forms of BMP-4 proteins, further described below, are being expressed and secreted from the stable CHO lines.

EXAMPLE VII

Characterization and Biological Activity of Expressed BMP-2 and BMP-4

To measure the biological activity of the expressed BMP-2 and BMP-4 proteins obtained in Example VI above, the proteins

are recovered from the cell culture and purified by isolating the BMP-2 and BMP-4 proteins from other proteinaceous materials with which they are co-produced as well as from other contaminants. The purified protein is assayed in accordance with the rat bone formation assay described in Example III using a modified scoring method described below.

A. COS Expressed Protein

The COS expressed material of Example VI may be partially purified on a Heparin Sepharose column. 4 ml of the collected post transfection conditioned medium supernatant from one 100 mm culture dish is concentrated approximately 10 fold by ultrafiltration on a YM 10 membrane and then dialyzed against 20mM Tris, 0.15 M NaCl, pH 7.4 (starting buffer). This material is then applied to a 1.1 ml Heparin Sepharose column in starting buffer. Unbound proteins are removed by an 8 ml wash of starting buffer, and bound proteins, including BMP-2 polypeptides, are desorbed by a 3-4 ml wash of 20 mM Tris, 2.0 M NaCl, pH 7.4.

The proteins bound by the Heparin column are concentrated approximately 10-fold on a Centricon 10 and the salt reduced by diafiltration with 0.1% trifluoroacetic acid. The appropriate amount of this solution is mixed with 20 mg of rat matrix and then assayed for <u>in vivo</u> bone and/or cartilage formation activity by the Rosen-modified Sampath - Reddi assay. A mock transfection supernatant fractionation is used as a control.

The implants containing rat matrix to which specific amounts of human BMP-2 or BMP-4 have been added are removed from rats after seven days and processed for histological evaluation. Representative sections from each implant are stained for the presence of new bone mineral with von Kossa and acid fuschin, and for the presence of cartilage-specific matrix formation using toluidine blue. The types of cells present within the section, as well as the extent to which these cells

display phenotype are evaluated and scored as described in Example III.

Addition of the expressed human BMP-2 or BMP-4 to the matrix material results in formation of cartilage-like nodules at 7 days post implantation. The chondroblast-type cells are recognizable by shape and expression of metachromatic matrix. The amount of activity observed for human BMP-2 or BMP-4 indicates that it may be dependent upon the amount of human BMP-2 or BMP-4 protein added to the matrix sample.

Similar levels of activity are seen in the Heparin Sepharose fractionated COS cell extracts. Partial purification is accomplished in a similar manner as described above except that 6 M urea is included in all the buffers.

B. CHO Expressed Protein

(1) BMP-2

To measure the biological activity of the BMP-2 proteins expressed in accordance with Example VIB above, .5 liters of conditioned media is directly adsorbed to 1 ml Heparin Sepharose (Pharmacia) column. The resin is washed with 0.15 M NaCl, 6.0 M urea, 20 mM Tris, pH 7.4 and then developed with a linear gradient to 1.0 M NaCl, 6.0 M urea, 50 mM Tris, pH 7.4. Fractions are assayed by the rat ectopic cartilage and bone formation assay described in Example III. The highest specific activity fractions are pooled and concentrated by ultrafiltration on a YM-10 (Amicon) membrane. Conditioned medium from CHO cells not transfected with the BMP-2 gene is prepared similarly, except that a step gradient to 1 M NaCl is used. Protein concentration is determined by amino acid analysis.

Further purification is achieved by preparative $NaDodSO_4$/PAGE [Laemmli, Nature 227: 680-685 (1970)]. Approximately 300 μg of protein is applied to a 1.5-mm-thick 12.5% gel: recovery is estimated by adding L-[^{35}S]methionine-labeled BMP-2 purified over heparin-Sepharose as described above. Protein is visualized by copper staining of an adjacent

lane [Lee, et al., Anal. Biochem. 166:308-312 (1987)]. Appropriate bands are excised and extracted in 0.1% $NaDodSO_4$/20 mM Tris, pH 8.0. The supernatant is acidified with 10% CF_3COOH to pH 3 and the proteins are desalted on 5.0 x 0.46 cm Vydac C_4 column (The Separations Group, Hesperia, CA) developed with a gradient of 0.1% CF_3COOH to 90% acetonitrile/0.1% CF_3COOH.

The pooled material is analyzed by SDS-PAGE using a 12% acrylamide [U.K. Laemmli, Nature 227:680 (1970)] stained with silver [R.R. Oakley, et al. Anal. Biochem. 105:361 (1980)] and by immunoblot [H. Towbin, et al. Proc. Natl. Acad. Sci. USA 76:4350 (1979)] of 13.5% gel. SDS-PAGE reveals that multiple molecular size forms of BMP-2 proteins are being expressed and secreted from the stable CHO lines. Under non-reduced conditions, the major protein species is represented by a broad band at 30,000 daltons. Lower molecular weight species are seen as well as higher species, most notably 82,000 daltons and 113,000 daltons.

The 30,000 dalton band reacts with a rabbit antiserum directed against an E. coli produced fragment of BMP-2 amino acids #130-#396 as shown in Figure 2 (SEQ ID NO: 4), with which it was incubated followed by ^{125}I-Protein A. Under reduced conditions the 30,000 dalton material shifts to the 16,000-20,000 range with several species within this range observed. Each band is recognized by a turkey-derived anti-peptide antibody directed against amino acids #350-#365 as shown in Figure 2 with which it is incubated followed by ^{125}I-rabbit anti-turkey IgG, as well as the anti-BMP-2 antibody described above. The peptide antibody is generated by coupling to bovine serum albumin with glutaraldehyde [J.P. Briand, et al. J. Immunol. Meth. 78:59 (1985)] in the presence of 100 ug/ml albumin. The broadness of the 30,000 dalton band and the mutiplicity of its subunits are contemplated to arise from differences in carbohydrate in the potential N-gylcosylation site or from N-terminal heterogeneity.

A major N-terminal amino acid sequence beginning at amino

acid #283 (Gln,Ala,Lys...) as shown in Figure 2 is obtained from the 30,000 dalton band isolated under non-reducing conditions. The calculated subunit molecular weight of a protein of amino acids 283-396 is approximately 13,000 daltons. Preliminary experiments indicate that over 90% of the biological activity in the total protein pool is eluted from a non-reduced SDS-PAGE at a relative mass of 30,000 daltons. It is contemplated therefore that a dimer of amino acids #283-396 of BMP-2, (referred to as a mature BMP-2) accounts for the majority of the biological activity in the mixture of expressed BMP-2 proteins. It is further contemplated that processing of BMP-2 to the mature forms involves dimerization of the proprotein (amino acids #24 Leu, Val, Pro . . . to #396) and removal of the N-terminal region in a manner analogous to the processing of the related protein TGF-β [L.E. Gentry, et al. <u>Molec. & Cell Biol.</u> <u>8</u>:4162 (1988); R. Dernyck, et al. <u>Nature</u> <u>316</u>:701 (1985)].

Immunoblot analysis using antibodies directed against a portion of the mature region (amino acids #350-365) and an antibody directed against the pro region (amino acids #103-116) of the 82,000 and 113,000 higher molecular weight species of BMP-2 under both non-reduced and reduced conditions suggests that these species may represent intermediate forms in the processing of the BMP-2 dimer. A 66,000 dalton species is present under reduced conditions. The 113,000 dalton species is contemplated to comprise proprotein dimers of 113,000 daltons (2 subunits of 66,000 daltons) and the 82,000 dalton species is contemplated to comprise a proprotein subunit linked to a mature BMP-2 subunit (66,000 daltons plus 18,000 daltons). Based on these analyses, approximately 50% of the total protein is active mature BMP-2.

The pool of protein containing recombinant human BMP-2 is assayed in accordance with the rat cartilage and bone formation assay described in Example III using a modified scoring method as follows, three non-adjacent sections are evaluated from each

implant and averaged. "+/-" indicates tentative identification of cartilage or bone; "+1" indicates >10% of each section being new cartilage or bone; "+2", >25%; "+3", >50%; "+4", ~75%; "+5", >80%. A "-" indicates that the implant is not recovered. The scores of the individual implants (in triplicate) are tabulated to indicate assay variability. BMP-2 protein is implanted subcutaneously in rats for times ranging from 5-21 days and the resulting implants evaluated histologically for the presence of newly formed cartilage and bone. Additionally, the level of alkaline phosphatase, synthesized by both cartilage and bone cells is measured.

Addition of partially purified CHO expressed human BMP-2 to the matrix material induces both new cartilage and new bone formation. Implantation of amounts of 0.46 - 115.3 μg of protein tested for times ranging from 5-21 days results in the induction of new cartilage and bone formation. Induction of cartilage formation is evident by day 7 and induction of bone formation is evident by day 14 for the lowest dose. The time at which bone formation occurs is related to the amount of BMP-2 implanted. At high doses bone can be observed at five days.

The development of cartilage and bone with time of a 12.0 microgram dosage of protein containing BMP-2 is summarized below. Amounts of new cartilage and bone are evaluated semi-quantitatively and scored on a scale of 0 to 5. Individual implants are listed to illustrate assay variability. At 5 days, many immature and some hypertrophic cartilage cells are present in the BMP-containing implant, but no mineralizing cartilage is detected. After 7 days chondrogenesis progresses so that most of the cartilage cells are hypertrophic and surrounded by mineralized matrix. Osteoblasts appear to be actively secreting osteoid, which is not yet mineralized. Day 7 implants have the greatest alkaline phosphatase content reflecting production by both chondrocytes and osteoblasts. Vascular elements, including giant cells and bone marrow precursors, are seen and are most abundant in areas where

calcified cartilage is undergoing remodeling.

The decline of alkaline phosphatase activity on day 10 signals the end of chondrogenesis in the implants. At 14 days the removal of calcified cartilage is nearly complete and bone is widespread. Osteoblasts and osteoclasts are abundant and appear to be actively engaged in the organization of newly formed trabecular bone. The levels of alkaline phosphatase reflect osteoblast activity at this stage in the maturation process. The vascularity of the implants has increased markedly, and hematopoietic cell maturation is tentatively observed.

At 21 days, implants show increased maturity over the previous time point. The bone is highly organized with mature marrow spaces, and bone-forming cells embedded in mineralized bone matrix are apparent. At 21 days, all remnants of matrix carrier have been removed in contrast to the control implants with no BMP, where matrix remains intact.

$NaDodSO_4$/PAGE is used to purify each of the three BMP-2 species to homogeneity. The overall recovery of BMP-2 protein after electrophoresis, desalting, and concentration is approximately 30% and 87% of the BMP-2 is the 30,000 dalton form. All three forms of BMP-2 show in vivo activity when assayed for cartilage and bone induction. The 30,000 and 82,000 dalton species were equivalent in this assay while the 113,000 dalton species showed significantly less activity.

(2) BMP-4

To measure the biological activity of BMP-4 expressed in accordance with Example VIB above BMP-4 is collected from the conditioned medium by batch adsorbing BMP-4 to heparin sepharose CL-6B using 3ml swelled heparin sepharose per liter conditioned media (CM) and stirring overnight at 4°C. The heparin sepharose is collected by filtering the CM through a fitted glass filter and washed with cold (4°C) 50mM Tris pH 7.4. A Pharmacia column is packed with the heparin sepharose

using 50mM Tris buffer and washed with buffer to the baseline. Elution is carried out with, the following gradient of sodium chloride:

Buffer A: 50mM Tris pH 7.4

Buffer B: 50mM Tris pH 7.4, 1M NaCl

BMP-4 containing fractions are located using Western blots probed with antipeptide antibody W10 (an anti-peptide polyclonal antibody recognizing the carboxy terminus of BMP-2). The BMP-4 containing fractions are pooled, the NaCl concentration is adjusted to 0.8M, and the pool is loaded onto a Butyl Toyopearl hydrophobic interaction column. Gradient elution from the hydrophobic interaction column is carried out using a sodium chloride and ethanol gradient:

Buffer A: 50mM Tris pH 7,4, 0.8M NaCl

Buffer B: 50mM Tris pH 7,4, 10% Ethanol

BMP-4 elutes at approximately 0.37M NaCl, 5.4% ethanol. The BMP-4 containing fractions are pooled and concentrated. Yields are approximately 33μg/liter CM of >95% pure material.

SDS-PAGE and silver stain analysis reveals that BMP-4 typically migrates as a single band at approximately 35kD (non-reduced) and reduces to a single band at approximately 22kD. BMP-4 is, therefore, a dimer of approximate molecular weight 35kD which reduces to a monomer of approximate molecular weight 20kD. Monomers of 18 and 22kD have been detected. Monomer can also be seen reducing from a high molecular weight region (>67kD) where it is assumed to be associated with an unprocessed BMP-4 molecule. The 2D pattern indicates that heterodimers are formed between the various molecular weight monomeric species.

BMP-4 is sensitive to N-glycanase, Endoglycosidase H, and Endoglycosidase F digestion, indicating the presence of N linked high mannose sugars. In a western format, ConA and WGA bind BMP-4, again indicating the presence of N-linked high mannose glycans. Lentil Lectin, which indicates the presence of α-D mannosyl or α-D glycosyl linkages, also binds BMP-4.

N-terminal sequence analysis reveals a single amino terminus at serine #293. Indicating a cleavage site at amino acid 293. This amino terminus is analogous to the Gln, Ala, Lys... of BMP-2. Mature BMP-4 is, therefore, a dimer of amino acids #293 - #408 as shown in Figure 3 (SEQ ID NO:4).

Presently, experiments indicate a minimum dose of 156ng reproducibly induces cartilage formation with BMP-4 in the rat ectopic assay which is comparable to BMP-2. Bone that is formed by BMP-4 is highly calcified, organized, and histologically similar to that formed by BMP-2, as described above. The time course of the appearance and the subsequent remodelling into bone is similar for BMP-4 and BMP-2.

The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto.